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13. ABSTRACT (Maximum 200 Words)

Women who have familial breast cancer often have a germline mutation of the breast cancer susceptibility gene known as BRCA1. The function of BRCA1 is not totally understood. Previously, immunochemical analysis of a series of breast cancer cell lines demonstrated a correlation between the expression of $p27^{Kip1}$ and BRCA1. The $p27^{Kip1}$ is a member of the universal cyclin-dependent kinase inhibitor family. BRCA1 has a number of activities including DNA repair, growth inhibition, and as a transcription factor. Here we demonstrate that BRCA1 transactivates expression of $p27^{Kip1}$. This transactivation is dependent on the presence of a functional C-terminal transactivation domain. Promoter-deletion analysis identified the presence of a putative BRCA1-responsive element located at position -615 to -511 of the $p27^{Kip1}$ promoter. These results suggest that the transcriptional regulation of $p27^{Kip1}$ by BRCA1 may be a mechanism for BRCA1-induced growth inhibition. Future studies will focus on examining the expression of BRCA1 and $p27^{Kip1}$ in clinical breast tumor samples and in ovarian cancer cell lines. I will also determine if $p27^{Kip1}$ is regulated by BRAC2.

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Annual Report
Year 1
Interaction of BRCA1 and p27 (kip1) Pathway in Breast Cancer
PI: James O'Kelly

Award number: DAMD 17-02-1-0337

Introduction: Families with inherited breast and ovarian cancers frequently have mutations of the breast cancer susceptibility gene BRCA1 (Futreal *et al.*, 1994) The BRCA1 gene encodes a 220kDa nuclear protein whose precise biochemical function remains unclear, although multiple functions have been suggested. These include DNA repair, growth inhibition and as a transcription factor (Aprelikova *et al.*, 1999; Chapman and Verma, 1996; Chen *et al.*, 1999; Haile and Parvin, 1999).

Cell cycle progression is governed by a family of cyclin-dependent kinases, whose activity is regulated by phosphorylation, activated by cyclin binding and inhibited by various inhibitors, such as $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (Sherr, 1994; Sherr and Roberts, 1999). A number of studies have also examined $p27^{Kip1}$ expression in a series of tumors to determine if there is any diagnostic or prognostic significance. It has been shown that $p27^{Kip1}$ protein decreases during tumor development and progression in breast, colon, prostate and ovarian cancers (Catzevalos *et al.*, 1997; Ciaparrone *et al.*, 1998; Cordon-Cardo *et al.*, 1998; Masciullo *et al.*, 1999). Previously we observed a correlation between the expression of BRCA1 and $p27^{Kip1}$ in a series of breast cancer cell lines (Elstner *et al.*, 2001). In this study, we have analyzed the $p27^{Kip1}$ promoter in order to determine if this cyclin-dependent kinase inhibitor (CDKI) is transcriptionally activated by BRCA1, and to elucidate the elements important for this activity.

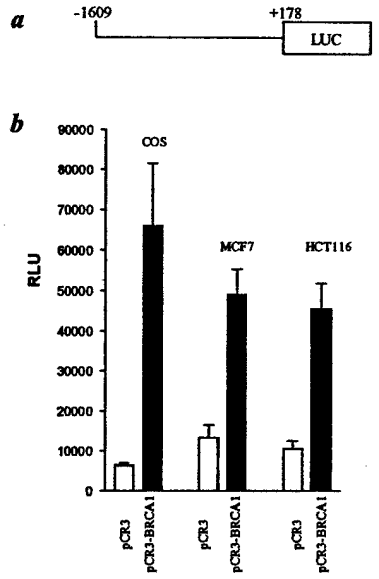

Figure 1 BRCA1 transactivates the mouse $p27^{kip1}$ promoter. a) Schematic representation of the $p27^{kip1}$ promoter luciferase promoter. b) COS, MCF7 and HCT116 were cotransfected with p27ptr-luc and either pCR3 or pCR3-BRCA1 and luciferase activity measured 48 h later. Results are shown as RLU which is the ratio of the luciferase units observed for the promoter construct compared to that seen for the pRL-SV40

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Task 1: Determine if BRCA1 can transactivate expression of $p27^{kip1}$: Using transient transfections I examined the effect of BRCA1 on both mouse and human $p27^{Kip1}$ promoter reporter gene expression in COS, MCF7 breast cancer and HCT116 colon cancer cells (**Figure 1a, b**). pCR3-BRCA1 activated the mouse $p27^{Kip1}$ promoter by 10-fold in COS cells and 5-fold in MCF7 and HCT116 cells (**Figure 1b**), as compared to the pCR3 vector. Similar fold activation was also observed for the human $p27^{Kip1}$ promoter in these cell lines (data not shown).

Task 2: Determine the specificity of $p27^{Kip1}$ induction by BRCA1 using various synthetic and tumor associated BRCA1 mutants: To investigate further the specificity of $p27^{Kip1}$ induction by BRCA1 I studied the effect of various synthetic and tumor-associated BRCA1 mutants on $p27^{Kip1}$ promoter reporter gene expression (**Figure 2a**). In these reporter assays, four different tumor-associated BRCA1 mutants and a synthetic BRCA1 mutant (del 500-1863) were unable to significantly transactivate the $p27^{Kip1}$ promoter reporter compared to the wild-type BRCA1 (**Figure 2b**). However, a second synthetic mutant lacking only the RAD51-interacting domain but with a functional nuclear localization signal and C-terminal transactivation domain, BRCA1 (del 515-1091), was able to transactivate the $p27^{Kip1}$ promoter nearly as efficiently as the wild-type BRCA1 (**Figure 2b**).

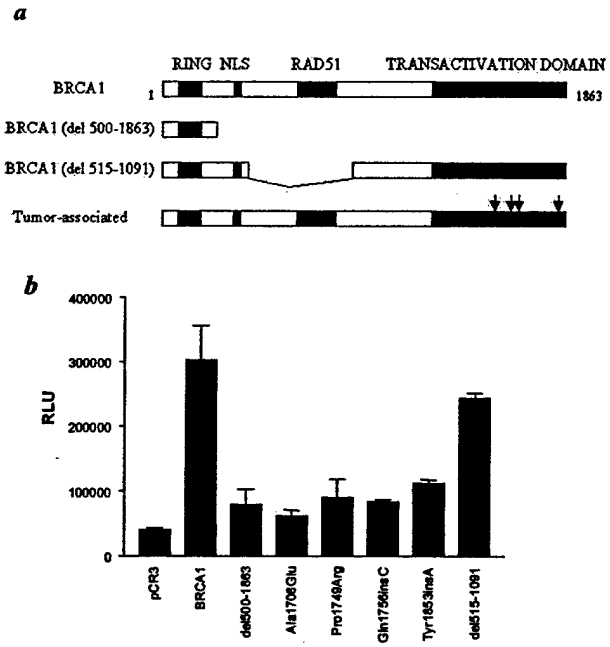

Figure 2. BRCA1 mutants lacking the C-terminal transactivation domain are defective for activation of $p27^{kip1}$. a) Schematic representation of the BRCA1 mutants indicating the important domains for BRCA1 function. Arrows indicate the position of the C-terminal mutations of the BRCA1 gene. B) COS cells were co-transfected with p27ptr-luc and pCR3 or either wild-type or mutant pCR3-BRCA1 expression plasmids as indicated

I next investigated the possible mechanism for regulation of $p27^{Kip1}$ by BRCA1. It has been shown in other studies that the regulation of expression of $p27^{Kip1}$ is primarily via post-translational mechanisms. Western immunoblot analysis of HCT116 cells demonstrated that endogenous $p27^{Kip1}$ protein expression was up-regulated by wild-type BRCA1 but not by either the control vector pCR3 or a mutated BRCA1 (Gln1756insC) (**Figure 3a**). Wild-type and mutant forms of BRCA1 were expressed at similar levels (**Figure 3a**), indicating that BRCA1 up-regulates $p27^{Kip1}$ protein expression.

To determine the mechanism for this up-regulation (transcriptional versus post-translational) I isolated RNA from cells transiently transfected with control vector, wild-type BRCA1 or mutated BRCA1 and proceeded with reverse transcription-PCR. PCR for BRCA1 used primers for exons 14 and 15 so that both wild-type and mutant BRCA1 could be detected. At 25 cycles a PCR product for both BRCA1 constructs was observed (**Figure 3b**). However, at 30 cycles a PCR product for $p27^{Kip1}$ was detected only in the cells transfected with wild-type BRCA1 (**Figure 3b**). By 35 cycles the $p27^{Kip1}$ PCR product was equivalent in all samples (data not shown). Thus these results suggest that the regulation of $p27^{Kip1}$ by BRCA1 is transcriptional.

Task 3: Localize the BRCA1 response element in the promoter region of $p27^{kip1}$ and verify BRCA1 binding by EMSA: Experiments were carried out using deletion mutants of the mouse $p27^{Kip1}$ promoter. Deletion up to position -774 increased the activity of BRCA1 on the $p27^{Kip1}$ promoter reporter constructs (**Figure 4a**), which is consistent with a previous report showing that the mouse $p27^{Kip1}$ promoter contains negative regulatory elements in the region -1609 to -925 (Kwon *et al.*, 1996). Further deletion to -615 did not significantly decrease the response of the $p27^{Kip1}$ promoter to BRCA1 (**Figure 4a**). However, BRCA1 responsiveness was lost by deletions up to position -511. These results suggested that a putative BRCA1-responsive element was located between positions -615 and -511 of the $p27^{Kip1}$ promoter.

BLAST analysis determined that this region of the mouse $p27^{Kip1}$ promoter (-774 to the translation start site) was 94% identical to this region of the human $p27^{Kip1}$ promoter, which starts at position -943.

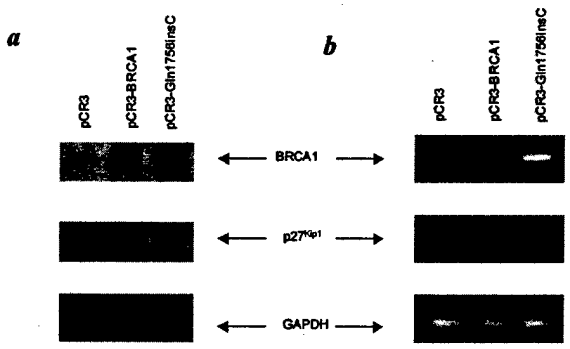

Figure 3. BRCA1 transcriptionally regulates $p27^{kip1}$ expression. a) HCT116 colon cancer cells were transfected with pCR3, pCR3-BRCA1 or pCR3-BRCA1 (gln1756insC). Proteins were harvested 48 h post-transfection and analyzed by SDS-PAGE and Western blot. Expression of endogenous $p27^{kip1}$ protein is up-regulated only in the presence of wild-type BRCA1. b) HCT116cells were transfected as in (a) and RNA harvested by Trizol 48 h post-transfection. RT-PCR demonstrated that both wild-type and mutant BRCA1 were being expressed. Expression of $p27^{kip1}$ was only observed in the cells transfected with wild-type BRCA1.

Subsequent transient transfection assays with deletion mutants of the human $p27^{Kip1}$ promoter demonstrated that the putative BRCA1-responsive element identified in the mouse $p27^{Kip1}$ promoter was located to the same region of the human $p27^{Kip1}$ promoter, position -784 to -680 (data not shown).

These results in conjunction with the immunoblot analysis of the breast cancer cell lines suggest that the effect of BRCA1 on $p27^{Kip1}$ is p53-independent. The correlation of BRCA1 and $p27^{Kip1}$ protein expression was observed in breast cancer cell lines expressing either wild-type p53 (MCF7) or mutant p53 (MDA-MB-231, T-47D). Also the putative p53 elements in the $p27^{Kip1}$ promoter are 5' to position -774, the $p27^{Kip1}$ promoter deletion construct having the greatest induction by co-transfection with BRCA1 (**Figure 4b**).

However these results do not determine whether $p27^{Kip1}$ activation by BRCA1 is a direct or indirect effect by BRCA1. To investigate the effect of BRCA1 on the $p27^{Kip1}$ promoter I generated oligonucleotides spanning the region of the mouse promoter containing the putative BRCA1-responsive element as identified by the promoter deletion analysis. These oligonucleotides (oligo A -609 to -575, oligo B -577 to -543, oligo C -545 to -511) were used in EMSA with nuclear lysates from two breast cancer cell lines MCF7 and HCC1937, a breast cancer cell line which is unizygous for the BRCA1 5382insC mutation, resulting in termination of BRCA1 protein translation at codon 1829 (Tomlinson *et al.*, 1998). Nuclear extract from MCF7 produced a slowly migrating band with oligo C but did not show any significant binding to oligo A or oligo B (**Figure 4b**). In contrast, nuclear extract from HCC1937 did not produce a band shift with any of the oligonucleotides (**Figure 4b and data not shown**). Subsequently I determined by immunohistochemistry that HCC1937 did express BRCA1 protein (**Figure 5b**).

The slowly migrating complex observed with MCF7 nuclear extract and oligo C could be competed 90% by a 10-fold excess of cold oligo C (**Figure 4b**). Some competition was observed with cold oligo A but there was no competition observed with an excess of an unrelated oligo (oligo U; STAT site from MUC1 promoter). This suggests that the DNA-protein complex is specific.

BRCA1 has been shown to interact with a number of other proteins. I used a number of antibodies in the EMSAs to determine the proteins involved in the slowly migrating complex. Of all the antibodies tested, only an antibody against exon 11 of BRCA1 resulted in a supershifting of the complex (Figure 4b). The antibody against the C-terminus of BRCA1 decreased the protein-DNA complex by 40-50%, suggesting that this antibody might interfere with BRCA1 binding to the DNA (data not shown). Together these results suggest that BRCA1 is interacting directly with the DNA sequence from the $p27^{Kip1}$ promoter.

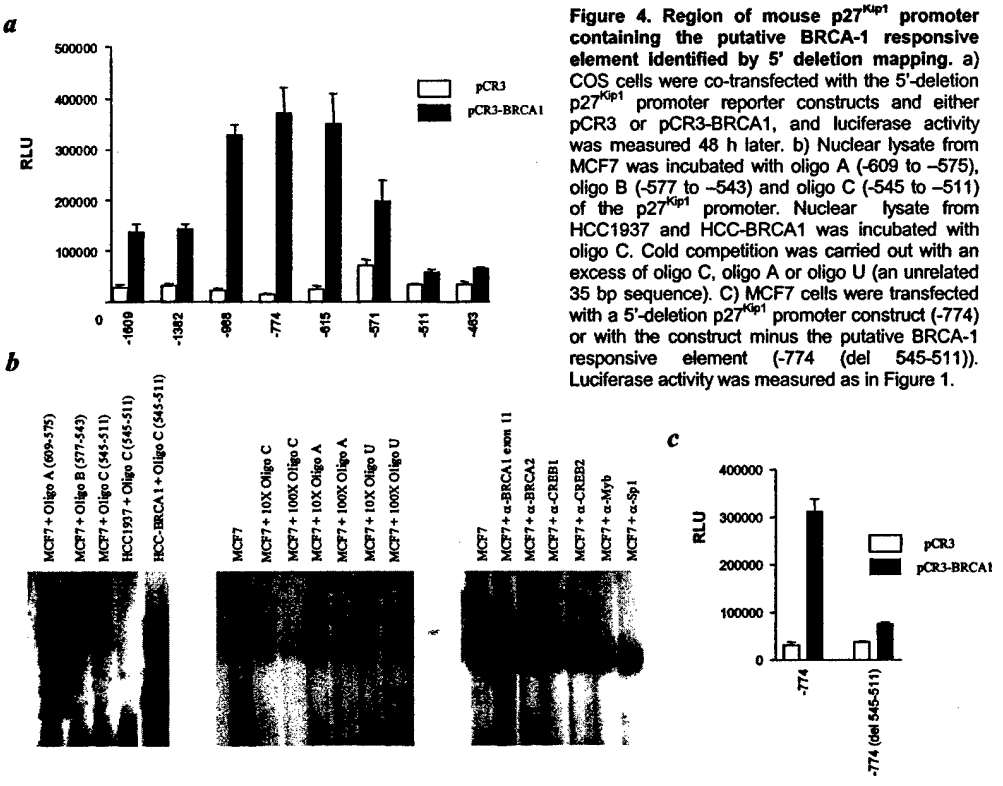

Figure 4. Region of mouse $p27^{Kip1}$ promoter containing the putative BRCA-1 responsive element identified by 5' deletion mapping. a) COS cells were co-transfected with the 5'-deletion $p27^{Kip1}$ promoter reporter constructs and either pCR3 or pCR3-BRCA1, and luciferase activity was measured 48 h later. b) Nuclear lysate from MCF7 was incubated with oligo A (-609 to –575), oligo B (-577 to –543) and oligo C (-545 to –511) of the $p27^{Kip1}$ promoter. Nuclear lysate from HCC1937 and HCC-BRCA1 was incubated with oligo C. Cold competition was carried out with an excess of oligo C, oligo A or oligo U (an unrelated 35 bp sequence). C) MCF7 cells were transfected with a 5'-deletion $p27^{Kip1}$ promoter construct (-774) or with the construct minus the putative BRCA-1 responsive element (-774 (del 545-511)). Luciferase activity was measured as in Figure 1.

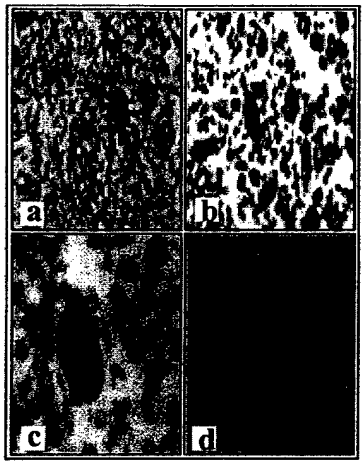

Figure 5. Expression of $p27^{Kip1}$ is decreased in cells expressing mutated BRCA1. a) and c), b) and d) Formalin-fixed paraffin-embedded MCF7 and HCC1937 cells, respectively. a) and b) N-terminal anti-BRCA1 at 4 x magnification. C) and d) Anti- $p27^{Kip1}$, 10 x magnification.

Since nuclear extract from the mutant BRCA1 cell line HCC1937 did not bind the DNA sequence from the $p27^{Kip1}$ promoter, I re-introduced wild-type BRCA1 into this cell line (HCC-BRCA1) and repeated the EMSA analysis. In this experiment a slowly migrating complex was observed with oligo C and nuclear extract from HCC-BRCA1 (**Figure 4b**). This slowly migrating complex with HCC-BRCA1 was less than that observed for MCF7 but the level of expression of wild-type BRCA1 in HCC-BRCA1 was lower than that observed for MCF7 (data not shown). Thus introduction of wild-type BRCA1 did restore binding to the $p27^{Kip1}$ promoter sequence. Since it appeared that the BRCA1-responsive element could be localized to a 35 bp region of the $p27^{Kip1}$ promoter, I generated a construct in which this region was deleted. Transient transfection assays using the deletion construct -774 and the construct without the putative BRCA1-responsive element -774 (del 545-511) demonstrated that the removal of this 35 bp region decreased BRCA1 responsiveness of the $p27^{Kip1}$ promoter by 80% (**Figure 4c**). Therefore it does appear that the BRCA1-responsive element is located at position -545 to -511 of the mouse $p27^{Kip1}$ promoter, which corresponds to -714 to -680 of the human $p27^{Kip1}$ promoter. This region is 100% identical between the mouse and human promoter. Further studies are continuing to define this region and determine whether other factors are involved with BRCA1 regulation of $p27^{Kip1}$.

Task 4: Correlate expression of BRCA1 and $p27^{Kip1}$ in a large series of breast cancers and find associations with clinical features and outcomes. We have obtained a series of 75 breast tumor samples collected previously, from the Department of Epidemiology at the University of Southern California. The samples were derived from patients with early onset bilateral breast cancer and known BRCA1 mutation status as well as other clinical parameters previously determined. I will analyze each sample with N- and C-terminal BRCA1 antibodies, $p27^{Kip1}$ and $p21^{Waf1}$ antibodies.

Task 5 Determine if BRCA1 induces expression of $p27^{Kip1}$ and $p21^{waf1}$ in ovarian cancer cells: I am currently beginning to investigate expression of BRCA1, $p27^{Kip1}$ and $p21^{waf1}$ in ovarian cancer cell lines.

Task 6 Determine if $p27^{Kip1}$ is regulated by breast cancer susceptibility gene 2 (BRCA2): I have performed deletion analysis of the $p27^{Kip1}$ promoter in transient transfection assays suggests that BRCA2-responsiveness is in the region –988 to –925. I am currently making constructs to delete this region from the full-length promoter. Possible further experiments are gel shift/EMSA analysis to demonstrate BRCA2 binding and identification of other co-factors which may be involved with BRCA2.

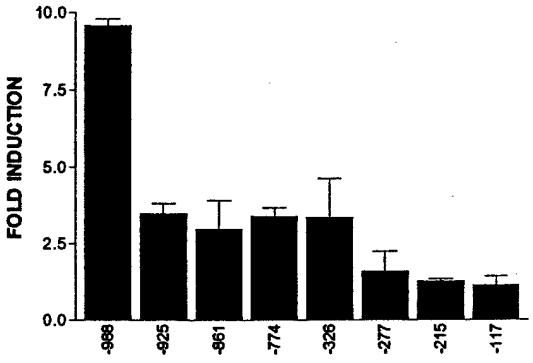

Figure 6. Deletion analysis of the $p27^{Kip1}$ promoter. HCT116 cells were transfected with p27ptr-luc and pCR3 with either wild-type of mutant pCR3-BRCA2 expression plasmids as indicated. Luciferase activity was measured 48 h post-transfection. BRCA2 responsiveness is in the region –988 to -925

Key Research Accomplishments:

- BRCA1 was shown to transcriptionally activate the CDI $p27^{Kip1}$
- Four-different tumor associated BRCA1 mutants and a synthetic BRCA1 mutant were unable to transactivate $p27^{Kip1}$
- The region of the $p27^{Kip1}$ promoter containing a putative BRCA1 responsive element was mapped to positions -545

to -511 of the mouse or positions -714 to -680 of the human $p27^{Kip1}$ promoter.

Reportable Outcomes: None to date

Conclusions:

Wild-type BRCA1transactivates $p27^{Kip1}$ via the BRCA1 response element located between positions -545 to -511 of the mouse $p27^{Kip1}$ promoter corresponding to positions -714 to –680. The decrease in $p27^{Kip1}$ expression noted in breast tumors might be a reflection of a loss of functional BRCA1, resulting either from a mutation in the BRCA1 gene or by methylation of the BRCA1 promoter.

So what?

Loss of functional BRCA1 might be expected to result in impaired growth inhibition due to ineffective regulation of $p27^{Kip1}$. Therefore, understanding the mechanisms controlling $p27^{Kip1}$ expression in breast tumors may provide new strategies to inhibit tumor growth.

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